Reduction of the immunogenicity of non-human grafts

The present invention relates to a transplantation material produced by dissociation of porcine tissue and removal of macrophages and/or microglial cells therefrom. The tissue could be an embryonic or fetal neural tissue and the removal could be effected by exposing the preparation above to antibodies against the Galα1-3Galβ1-R epitope and a complement reagent. The invention also relates to the use of such a transplantation material for preparing a pharmaceutical preparation useful when transplanting neural tissue. The invention also relates to a kit comprising one or more enzymes for tissue dissociation, an antibody preparation and a complement reagent. The invention also relates to a process for removal of macrophages and/or microglial cells from porcine embryonic or fetal neural tissue. Finally, the invention relates to a process for treatment of neurological disorders with such pretreated porcine neural grafts.

Background of the invention

Neural transplantation is a potential therapy for Parkinson's and Huntington's diseases (Peschanski et al., 1995; Olanow et al., 1997; Borlongan et al., 1999; Dunnett and Björklund, 1999). Due to the short supply of human donor material (Dunnett and Björklund, 1999), tissue derived from an animal source is considered an appropriate alternative (Isacson and Breakefield, 1997; Edge et al., 1998), and has the advantage of being genetically modifiable (Cozzi and White, 1995; Zawada et al., 1998). When implanted into the striatum of immunosuppressed rats, ventral mesencephalic tissue from pig embryos can grow anatomically correct connections (Isacson et al., 1995) and restore induced behavioural deficits (Huffaker et al., 1989; Galpern et al., 1996). So far, porcine neural tissue has been grafted to 12 Parkinson and 12 Huntington patients in small-scale safety trials (Edge et al., 1998).

Porcine neural grafts are, however, prone to immune-related rejection in the human brain (Deacon et al., 1997). Rejection of murine neural grafts can be prevented by

treatment of rat hosts with antibodies against T lymphocytes of the immune system (Okura et al., 1997). The human T-cell repertoire includes cells that are activated by porcine major histocompatibility complex (MHC) antigens on porcine cells (direct recognition), and cells that are activated by peptides derived from porcine proteins after processing and presentation by human antigen presenting cells (indirect recognition) (Satake et al., 1994; Dorling et al., 1996). For direct recognition to occur, the porcine donor cells must express class I and/or class II MHC antigens, which are recognised by CD8 and CD4 T lymphocyte cells, respectively. Survival of human neural tissue in mouse brain can be prolonged by depletion of host CD4, but not CD8, T cells (Wood et al., 1996), proving that CD4 T cells are essential for neural xenograft rejection, which is also the case for rejection of non-neural xenografts (Pierson et al., 1989; Kaufman et al., 1995; Korsgren, 1997). In vitro, pig MHC class II antigens elicit a much stronger proliferative response in human T cells than pig MHC class I antigens (Kirk et al., 1993; Kumagai-Braesch et al., 1993; Yamada et al., 1995; Dorling et al., 1996), suggesting that cells which express MHC class II antigens, or have the potential to express these molecules once implanted, contributes heavily to the immunogenicity of the donor tissue. MHC class II antigens have not been reported to occur on neurons (Lampson, 1995).

Cyclosporin, an immunosuppressant that inhibits alloreactivity, is inadequate at protecting porcine xenografts from immune attack in rats (Wennberg et al., 1995). Continuous cyclosporin treatment of rats hosting porcine neural grafts resulted in some degree of graft survival in 74% of the animals 3-4 months after transplantation (Pakzaban et al., 1995). A Parkinson patient that received porcine neural grafts and continuous cyclosporin treatment had poorly surviving grafts 7.5 months after transplantation, considering that 1.2×10^7 cells were implanted and only 638 dopaminergic cells were present at postmortem histology (Deacon et al., 1997). In the clinical setting, the immunosuppression must, therefore, be more aggressive, which may have serious consequences in terms of side effects (Borlongan et al., 1996), or supplemented by reduction of donor tissue immunogenicity prior to transplantation.

WO9637602 A1 relates to the use of hepatocytes to treat subjects with disorders characterised by insufficient liver function. Methods are disclosed in order to make these hepatocytes less immunogenic, especially by masking certain antigens, in particular MHC class I antigen. The possibility to remove the Gal(α1,3)Gal epitope with α-galactosidase is also discussed, but there is no coupling of an expression of this epitope on macrophages and/or microglial cells and to remove these cells by using the Gal(α1,3)Gal epitope. Nothing is mentioned about neural cells.

WO9636358 A1 relates to reagents and methods in order to inhibit antibody-mediated xenograft rejection by xenogeneic transplant recipients. The object is to block antigen on endothelial cells of the donor organ i e cells being the donor cells at first brought in contact with the blood of the recipient. This document only relates to antibody-mediated immunity and the inhibition thereof by using anti-donor antibodies, whereas the present invention relates to the removal of a specific cell population from a neural cell mixture in order to make it less prone to stimulate the T cells of the recipient. Nothing is mentioned about neural cells and the Gal epitope is only mentioned as a possible antigen to block. Claim 1 of WO9636358 A1 is very broad and relates to the inhibition of all possible xenogeneic antigens. The largest difference compared to the process according to the present invention is that the latter relates to depletion of macrophages and/or microglial cells via the Gal epitope and not to blocking thereof.

Koulmanda, M. et al., Xenotransplantation 2(4), p 295-305 (1995) relates to a method for depletion of immunogenic cells from pancreatic tissue after transplantation. A requirement for that is cultivation in 90% O_2. The expression of the Gal epitope was studied in this pancreatic tissue as this epitope is important for rejection by being recognized of human antipig antibodies. Nothing is mentioned about neural tissue or expression of the Gal epitope on macrophages and the possibility to use the Gal epitope to deplete this cell population. In this study, anti-CD4 treatment of the recipient has been used to immunosuppress the recipient.

Summary of the invention

It has now been shown that when dissociated brain tissue from pig embryos is grown in primary culture, its macrophages and/or microglial cells become autofluorescent, express both classes of MHC antigens and induce proliferation of human T lymphocytes, whereas its astrocytes remain non-autofluorescent, express only MHC class I antigens and generate only a weak proliferative response in human T cells (Brevig et al., 1999). In attempts to remove macrophages and/or microglial cells from porcine brain tissue, a potential target according to the invention is the carbohydrate epitope, Galα1-3Galβ1-R (α-galactosyl epitope), which the inventors have now demonstrated on macrophages and/or microglial cells and endothelial cells, but not on neurons and astrocytes, in embryonic pig brain (Sumitran et al., 1999). Human serum contains a high titer of naturally occurring, complement-activating IgG and IgM antibodies against the α-galactosyl epitope (anti-Gal) (Galili, 1993; Rother and Squinto, 1996), but also antibodies against other epitopes expressed in ventral mesencephalon of pig embryos (Sumitran et al., 1999). Purified anti-Gal and complement may, therefore, have the ability to selectively lyse macrophages and/or microglial cells and endothelial cells, and remove these cells from dissociated porcine neural tissue prior to transplantation, for the treatment of neurological disorders.

As described above, neural xenograft survival is compromised by host CD4 T lymphocytes. In order to reduce the immunogenicity of porcine embryonic brain tissue, which is of interest as donor material in therapeutic xenografting for Parkinson's disease, Huntington's disease, multiple sclerosis, epilepsy, stroke, pain, and spinal cord injuries, the possibility of using the α-galactosyl epitope as target for antibody- and complement-mediated removal of macrophages and/or microglial cells has been studied in vitro by the inventors. After exposing brain cells isolated from 27-day-old pig embryos to anti-Gal and complement, the remaining cells were (i) analysed by flow cytometry to determine the content of macrophages and/or microglial cells, and (ii) co-cultured with human peripheral blood CD4 T lymphocytes to determine their ability to elicit a proliferative T-cell response, which was assessed by

measuring incorporation of tritiated thymidine. Incubation of porcine embryonic brain cells with normal human serum, which contains a high titre of anti-Gal, followed by rabbit complement reduced the number of macrophages and/or microglial cells, both when applied to freshly isolated cells and to cells grown in short-term primary culture. Cultured porcine embryonic brain cells treated with purified human anti-Gal and rabbit complement contained 0.8% macrophages and/or microglial cells and did not induce proliferation of human CD4 T cells, while brain cells given a control treatment with medium and rabbit complement contained 3.4% macrophages and/or microglial cells and induced a significant proliferative response in human CD4 T lymphocytes.

Detailed description of the invention

One object of the invention is a transplantation material, characterised in that it has been produced by

(a) dissociation of porcine embryonic or fetal neural tissue,

(b) removal of macrophages and/or microglial cells by exposing the preparation of step (a) to antibodies against the Galα1-3Galβ1-R epitope and a complement reagent.

Dissociation of a tissue could be either partial or full. Partial dissociation means dissociation of tissue into a suspension containing cell aggregates. Full dissociation means dissociation of tissue into a single-cell suspension. It is preferred that the partial or full dissociation is effected by the use of one or more enzymes such as proteases and/or deoxyribonucleases. Some examples of such enzymes are given by Barker et al. (1995) such as bovine trypsin from Worthington Biochemical Corporation. Another suitable enzyme is porcine trypsin, type IX, from Sigma. Some examples of media for washing the dissociated tissue are given by Watts et al. (1998) such as Dulbecco's modified Eagle's medium (DMEM). Phosphate buffered saline (PBS) could also be used. It is also preferred that the complement reagent is rabbit serum or complement purified from rabbit serum.

Examples of antibodies according to the invention are monoclonal or polyclonal antibodies. The antibodies may derive from any species, but are preferably human. The term monoclonal antibody is art-recognised terminology. Monoclonal antibodies of the present invention can be prepared according to general methods as described in Kohler et al., 1975. Polyclonal antibodies can, e.g., be purified from animal or human serum. There is also a possibility to create new molecules that binds to the epitope by means of computer simulation. Methods for computer simulation are known by a person skilled in the art, e.g., as described in EP 0660 210 A2.

The antibodies according to the invention bind to Galα1-3Galβ1-R-terminated epitopes. An example of such an epitope is the Galα1-3Galβ1-4GlcNAc-R epitope. The Galα1-3Galβ1-R antigen and the isolation thereof is described in Subcellular Biochemistry, Vol. 32: alpha-Gal and Anti-Gal, edited by Galili and Avila. Kluwer Academic/Plenum Publishers, New York, 1999; Liu et al. (1997); and Hallberg et al. (1998).

Another object of the invention is the use of the above-mentioned transplantation material for preparing a pharmaceutical preparation which is useful when transplanting neural tissue.

Another object of the invention is a kit, for use in treating a porcine tissue in order to reduce its immunogenicity, characterised in that it comprises one or more enzymes for tissue dissociation, a preparation of an antibody against the Galα1-3Galβ1-R epitope, and a complement reagent.

It is preferred that the antibody is a polyclonal antibody. It is especially preferred that the antibody is of human origin. It is also preferred that the antibody binds to macrophages and/or microglial cells. Examples of enzymes and complement reagents are given above.

Another object of the invention is a process for removal of macrophages and/or microglial cells from porcine embryonic or fetal neural tissue, characterised in that

(a) the neural tissue is dissociated and treated with an antibody against the Galα1-3-Galβ1-R epitope

(b) the macrophages and/or microglial cells are depleted from the preparation of step (a) by exposing the preparation in step (a) to the antibody coupled to a carrier or by flow sorting, or

(c) the macrophages and/or microglial cells are depleted from the preparation of step (a) by treating the preparation of step (a) with a complement reagent.

It is preferred that the porcine embryonic or fetal neural tissue is dissociated by the use of one or more enzymes such as proteases and/or deoxyribonucleases. It is also preferred that the antibody is a polyclonal antibody such as an antibody of human origin. It is also preferred that the complement is rabbit serum or complement purified from rabbit serum. Examples of suitable carriers are paramagnetic beads or a plastic surface.

It is preferred to dissociate the donor material and use antibody- and complement-mediated lysis, because even macrophages and/or microglial cells within small cell clusters can be removed by this mechanism.

It is also possible to convert the donor material into a single-cell suspension and physically remove macrophages and/or microglial cells e.g. by panning, paramagnetic beads or flow sorting although this may lower the amount of usable nerve cells. See Current Protocols in Immunology, edited by Coligan, J.E. et al. 1993, vol. 1-3, John Wiley & Sons, Inc.

Another object of the invention is a process for treatment of neurological disorders, characterised in that

(a) porcine embryonic or fetal neural tissue is dissociated,

(b) the dissociated tissue is treated with antibodies against the Galα1-3Galβ1-R epitope and a complement reagent in order to remove macrophages and/or microglial cells,

(c) the dissociated and antibody- and complement-treated tissue is transplanted into the human body.

Preferably, the disorders are selected from the group consisting of Parkinson's disease, Huntington's disease, multiple sclerosis, epilepsy, stroke, pain, and spinal cord injuries.

Instead of transplanting whole suspensions prepared from porcine brain tissue, the inventors propose that the dissociated tissue should be pretreated with anti-Gal and complement, the effect of which is shown in Figure 12. If the pretreatment with anti-Gal and complement can enhance survival of porcine neural grafts in humans, it may serve as a simple and inexpensive way of reducing donor tissue immunogenicity prior to clinical neural xenotransplantation.

After neural xenotransplantation, both direct and indirect recognition of the graft may occur, but the relative strength of these two mechanisms is not known in the pig-to-man xenograft situation (Gill and Wolf, 1995; Auchincloss and Sachs, 1998). The inventors have focused on direct recognition, but the pretreatment with anti-Gal and complement may also reduce indirect recognition (Brevig et al., in press). The effect of indirect recognition may be explained by the removal of cells and thus proteins carrying the α-galactosyl epitope, which can be opsonized by host anti-Gal for endocytosis, processing and presentation by host antigen-presenting cells. Because neurons may be recognized by T cells via the indirect pathway, porcine neural xenografts cannot be made completely non-immunogenic.

Short description of the figures

Figure 1. Flow cytometric analysis of unstained brain cells from 28-day-old pig embryos.

Figure 2. Expression of CD18, CD56, CD44, CD4, and GFAP by cells isolated from 28-day-old embryonic pig brains and grown for 14 days in primary culture.

Figure 3. Phagocytosis of *Escherichia coli* by cultured cells from 28-day-old embryonic pig brains.

Figure 4. Flow sorted astrocyte and macrophage microglial populations, separated on the basis of CD56 expression.

Figure 5. Expression of α-galactosyl epitopes on suspended ventral mesencephalic cells and microvascular endothelial cells isolated from porcine liver.

Figure 6. Western blot analysis of BS-IB4 reactive, solubilized glycoprotein isolated from cell membranes.

Figure 7. Saggital section of the ventral mesencephalon of a 27-day-old pig embryo stained immunohistochemically for tyrosine hydroxylase.

Figure 8. Expression of the α-galactosyl epitope on cultured porcine embryonic brain cells.

Figure 9. Effect of human serum and rabbit complement on cultured and freshly isolated porcine embryonic brain cells.

Figure 10. Reactivity towards the α-galactosyl epitope of the antibodies purified from normal human serum using porcine thyroglobulin.

Figure 11. Proliferative response of CD4 T lymphocytes from two human individuals when co-cultured with pretreated porcine embryonic brain cells.

Figure 12. Summary of the effect of anti-Gal and complement on dissociated porcine brain tissue in the clinical situation and in an in-vitro model.

Figure 13. Effect of the pretreatment on porcine neural graft function in a rat model of Parkinson's disease.

Figure 14. Histology of a graft derived from porcine embryonic VM tissue pretreated with anti-Gal and complement.

The invention will now be described by reference to some non-limiting examples

As used herein, the following terms have the following meanings:

α-galactosyl epitope means Galα1-3Galβ1-R (where R is the rest of the molecule),
anti-Gal means antibodies against Galα1-3Galβ1-R (where R is the rest of the molecule),
BSA means bovine serum albumin,
DMEM means Dulbecco's modified Eagle's medium,
EDTA means ethylenediaminetetraacetic acid,
FCS means fetal calf serum,
FITC means fluorescein isothiocyanate,
FBS means fetal bovine serum,
Gal means D-galactose,
GlcNAc means D-N-acetylglucosamine,
Ig means immunoglobulin,
mAb means monoclonal antibody,
PBS means phosphate buffered saline,

PMSF means phenylmethane sulfonyl fluoride,
PLMEC means porcine liver microvascular endothelial cell,
RPE means R-phycoerythrin,
TBS means Tris-buffered saline,
TH means tyrosine hydroxylase,
TNF means tumour necrosis factor,
VCAM means vascular cell adhesion molecule, and
VM means ventral mesencephalon.

All technical and scientific terms used herein are, unless otherwise defined, intended to have the same meaning as commonly understood by one of ordinary skill in the art. By the expression "comprising", we understand including but not limited to. Techniques employed herein are those that are known to one of ordinary skill in the art unless stated otherwise. Publications mentioned herein are incorporated by reference.

Examples

Materials and methods

For Example 1 and 2 (See also for Example 4-7)

Porcine fetal brain cells. Brain cells were isolated from whole brain or ventral mesencephalon of 28, 35, 42, and 56 days old pig fetuses (Table 1). Brains isolated from each of the 12 litters of 6-15 fetuses were pooled in Gey's Balanced Salt Solution (Life Technologies, Scotland) and meninges were carefully removed. The brains were cut into small pieces, transferred to brain-cell medium (Eagle's Minimum Essential Medium with Earle's Salts supplemented with L-glutamine, amino acids, vitamins (all Life Technologies), penicillin, hexamycin, and sodium bicarbonate) with 20% heat-inactivated (56°C, 30 min) horse serum (Life Technologies), triturated with a flame-polished pipette, and finally filtered through a Nitex filter with 80-μm pores. The

filtrates were diluted in brain-cell medium with 20% horse serum to give total volumes of 10, 20, 30, or 50 ml per 28, 35, 42, or 56 days old brain, respectively.

The brain cells were analysed directly after isolation or after 12-14 days in primary culture. Cultures were prepared by adding 5.0 ml cell suspension, prepared as described above, to poly-D-lysine (Sigma, USA) coated culture flasks (50-ml flasks; Nunc, Denmark), followed by incubation at 37°C in a humidified atmosphere of 5% CO_2 in air. After 2 days, when the cells had attached, the medium was changed to brain-cell medium with 20% horse serum, and subsequently twice a week to brain-cell medium with 10% horse serum.

The cultured brain cells were detached by gently sweeping the bottoms of the culture flasks with a plastic pipette (culture flasks were checked by microscopy to ensure that all cells had detached), transferred to V-bottomed tubes (content of one flask to one tube) and centrifuged (100 g, 10 min). The supernatants were discarded, each tube was given either 500 µl or 500 µl lymphocyte medium and the cells were resuspended using a syringe with a needle of 0.5 mm internal diameter. The tubes were left for 15 min at room temperature to sediment cell clusters, and the supernatants with single cells in suspension were harvested and used for subsequent analyses.

Staining reagents. For immunofluorescence staining of the brain cells, the following primary antibodies and antibody-fluorochrome conjugates were used: mouse anti-MHC I IgG_{2a} (immunogen: pig thymus; 1:10; clone PT85A; Veterinary Medical Research and Development (VMRD), USA), mouse anti-MHC II IgG_{2a} (immunogen: thymus from pig and other species; 1:10; clone TH16B; VMRD), mouse anti-pig CD4 IgG_{2a} (1:1; clone PT90A; VMRD), mouse anti-pig CD44 IgG_{2a} (1:1; clone PORC24A; VMRD), mouse anti-human CD18 IgG_1 FITC (1:1; clone MHM23; DAKO, Denmark), mouse anti-human CD56 IgG_1 RPE (1:1; clone MY31; BD), or polyclonal rabbit anti-cow GFAP (1:10; DAKO). Goat anti-mouse immunoglobulins $F(ab')_2$ RPE (1:10; DAKO) was used as secondary reagent for the MHC antibodies and the antibodies against CD4 and CD44. Swine anti-rabbit immunoglobulins FITC (1:10;

DAKO) was used as secondary antibody for the GFAP antibody. Samples exposed to species- and isotype-matched irrelevant antibody (mouse IgG_{2a} negative control (1:1; DAKO) or mouse IgG_1 negative control FITC/RPE/Cy5 (1:1; DAKO)) or secondary antibody only were used as negative controls. According to the manufacturers, primary antibodies against antigens from other species than pig cross-reacted strongly with the corresponding pig antigens. Higher concentrations of antibodies than indicated did not give stronger staining. Staining reagents for demonstration of phagocytosis are given below.

Staining procedures. Brain cells from cultures derived from the same brains were pooled prior to staining to eliminate possible variability between cultures. Volumes of 100 µl cell suspension containing 1.0×10^6 freshly isolated or cultured brain cells were transferred to Falcon tubes (BD) and given 10 µl antibody. After 30 min in the dark at room temperature, the cells were washed twice in PBS (250 g, 5 min) and resuspended in 100 µl PBS for indirect immunostaining or 500 µl PBS for direct immunostaining. Samples for indirect immunostaining were given 10 µl secondary antibody, incubated and washed as described above, and resuspended in 500 µl PBS. Prior to staining for GFAP, the cells were fixed and permeabilized in the Falcon tubes by incubating them for 10 min in 2.0 ml FACS Lysing Solution (BD), and subsequently for 10 min in 0.5 ml FACS Permeabilizing Solution (BD).

For demonstration of phagocytosis, 0.5 mg fluorescein-labelled *Eschericia coli* BioParticles (Molecular Probes, USA) was added to the medium of brain-cell cultures. After 30 min of incubation at 37°C, the cells were washed twice in PBS, detached, dissociated and analysed by flow cytometry, fluorescence microscopy or confocal laser microscopy. Prior to receiving the *E. coli* BioParticles, cultures to be analysed by confocal laser microscopy were given 20 µl 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) stock solution (1.0 mg DiI per ml 99% ethanol), incubated for 2 h at 37°C and washed twice in brain-cell medium with 10% horse serum.

Flow cytometry. Brain cells were stained as described above, and 1.0×10^4 ungated events were acquired from each sample on a FACScan flow cytometer (BD). A forward-scatter threshold was set to exclude subcellular debris. The instrument settings were identical for all acquisitions, except for the fixed cells, where the forward-scatter threshold was reduced.

Confocal laser scanning microscopy. Drops of suspended brain cells were placed on microscopy slides and covered by coverslips for ordinary fluorescence microscopy. Suitable parts of the samples were then scanned by a Leica TCS 4D DM R confocal laser scanning microscope (Leica, Germany) in dual-channel mode using excitation wavelengths of 488 and 568 nm, detectors with fluorescein and rhodamine optical filter sets, and linear AD converting.

Flow sorting. In order to separate astrocytes and macrophages/microglial cells, single-cell suspensions of cultured brain cells, prepared as described above, were sorted at a rate of $2\text{-}3\times10^3$ cells/s on a FACSVantage cell sorter (BD) equipped with a Helium-Neon laser (488 nm), an FL1/FL2 beam splitter (560 nm), and FL1 (530 ± 15 nm) and FL2 (575 ± 13 nm) filters. Six times 10^7 brain cells, in 5.0 ml PBS with 2% FCS, were given 200 µl mouse anti-CD56 IgG_1 RPE, incubated for 30 min at 4°C, washed twice in CellWash with 2% FCS (250 g, 5 min) and sorted by setting rectangular gates in a green-fluorescence/orange-fluorescence plot to sort CD56-positive cells (astrocytes) into one tube, and autofluorescent, CD56-negative cells (macrophages/microglial cells) into another. The sample fluid was PBS with 2% FCS. FACSFlow (BD) was used as sheat fluid. A forward-scatter threshold was set to exclude subcellular debris.

The purity of the cell sorted populations was determined by flow cytometry without further staining of the cells. The viability of the macrophages/microglial cells was determined by flow cytometry after staining with propidium iodide. The viability of the astrocytes was determined by counting the blue cells and the total number of cells, 10 min after mixing aliquots of 0.4% trypan blue solution (Sigma) and cell suspension.

Mixed lymphocyte-brain cell culture. Mononuclear blood cells (lymphocytes and monocytes) from 3 healthy blood donors (H-3970, H-3977, and H-8053) were isolated from buffy coats by flotation and cryopreserved as earlier described (Brevig et al., 1997). Prior to use, the cells were thawed in a water bath (37°C), washed (500 g, 10 min) 3 times in RPMI (Life Technologies), and left for 24 h in *lymphocyte medium* (90% RPMI 1640 with HEPES, 10% heat-inactivated FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2.0 mM L-glutamine; all Life Technologies) at 37°C in a humidified atmosphere of 5% CO_2 in air. Volumes of 100 µl lymphocyte medium containing 2.0×10^5 human mononuclear blood cells (responders) were dispensed in wells of U-bottomed microtiter plates (Nunc). Unseparated brain cells, sorted brain cells, and allogeneic mononuclear blood cells (stimulators) were γ-irradiated (50 Gy), and 2.0×10^4 cells in 100 µl lymphocyte medium were added to wells with responders (3 or 5 wells per combination). Wells receiving responders and lymphocyte medium but no stimulators were used as negative controls (5 or 12 wells per combination). Each well was given 1.0 µCi [^{3}H]-thymidine (Amersham, UK) 24 h prior to cell harvest. After 4 days of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air, cells were harvested with a cell collection system (Skatron, Norway) and counted in a β-counter (2000CA Tri-Carb; Packard, USA).

Statistical analysis. The proliferation data were analysed by one-way analyses of variance (ANOVAs) and one-sided, unpaired *t* tests.

For Example 3 (See also for Example 4-7)

Cell culture. The immortalised human umbilical vein endothelial cell line, ECV304 (ATCC; CRL-1998), was cultured in DMEM/FBS. Porcine liver microvascular endothelial cells (PLMECs) were isolated from fresh pig livers obtained from the local slaughter house. The specimens were chopped into pieces in medium containing 0.05% (w/v) collagenase (cat. no. 1 088 793; Boehringer Mannheim, Germany) and 0.002% (w/v) DNase I type II from bovine pancreas (cat. no. D-4527; Sigma, USA),

followed by digestion at 37°C for 30-45 min. The cell suspension was filtered to remove undissociated tissue, washed three times with PBS, and cultured for 1 week in 0.2% gelatin-coated 25 cm^2 flasks (Falcon, USA) using the medium indicated below. Expanded cells were activated for 18 h with 100 U/ml human recombinant TNF-α (Genzyme, USA), incubated on a rock n' roller for 1 h at room temperature with 50 µg (1 mg/ml) of anti-pig VCAM antibodies (clone 5D11; Alexion Pharmaceuticals, USA), washed once with PBS, and incubated with 100 µl of paramagnetic goat anti-mouse dynabeads (Dynal, Norway) at 4 °C for 45 min followed by immunomagnetic selection on a magnet. Positively selected cells were washed three times with PBS and cultured in gelatin-coated, six-well tissue culture plates (Falcon) in DMEM containing 10% FBS (Gibco, USA), penicillin (100 IU/ml; Sigma) and streptomycin (100 µg/ml; Sigma), and 1 ml/100 ml medium of endothelial cell growth factor (cat. no. E-9640; Sigma). On the second day of culturing, the beads were washed away using PBS. The cells were allowed to grow to confluence before subculturing. Endothelial cells were characterised by morphology, and by flow cytometric phenotyping using anti-E-selectin (clone BBIG-E4; cat. no. BBA 16; R&D systems, U.K.) and anti-pig VCAM antibodies. More than 95 % of the cells were endothelia cells.

Isolation of pig embryonic ventral mesencephalic cells. Ventral mesencephalic (VM) regions were obtained from E26-27 pig embryos of the Pigham strain (derived from a cross between Swedish Landrace x Yorkshire sows and Hampshire boars; Nyholms Breeding Farm, Sweden). Pigs were bred and euthanized according to local and national animal ethics regulations. The VM cells contained approximately 10-15% dopaminergic cells, the rest being other neurons, glia, and endothelial cells. The tissue was dissected in Hank's balanced salt solution with 0.3 µM lazaroid U-83836E (Upjohn Inc., USA) (Nakao et al., 1994). A VM tissue specimen, about 2 x 2 x 1 mm was cut into 4-6 smaller pieces, and these were transferred to cold storage medium (Grasbon-Frodl et al., 1996) and shipped to Huddinge Hospital for analysis the same or the following day. The tissue was then triturated into a cell suspension by several passages through a pipette. Prior to shipping and *in vitro* analysis, the viability was checked using ethidium bromide.

Flow cytometric assays. Five times 10^5 VM cells in 50 µl PBS were incubated with 50 µl human AB serum, anti-Gal antibody-depleted AB serum or RPMI 1640 medium for 1 h at 22 °C. The cells were washed three times with PBS containing 0.1% sodium azide. Ten µl of 1:4 diluted FITC-conjugated $F(ab)'_2$ fragments of goat anti-human IgG or IgM antibodies (109-096-098 and 109-096-129, respectively; Jackson ImmunoResearch Labs, USA) were added, and the samples were incubated on ice in the dark for 25 min. The cells were washed and then analysed by flow cytometry. α-galactosyl epitope expression was estimated by incubating VM cells or PLMECs with biotinylated *Bandereia Simplicifolia* isolectin B4 (4 µg/ml; cat.no. L-2140; Sigma) followed by detection with FITC-conjugated avidin (cat.no. 55880; Organon Teknika, USA) diluted 1:2000 in 1 % BSA/PBS. Alternatively, 200 µl of rabbit serum (cat. no. 439665, Biotest AG, Germany) was added to tubes containing 5 x 10^5 VM cells in 50 µl PBS and 50 µl human AB serum, anti-Gal antibody-depleted AB serum or RPMI 1640 medium and incubated again, now for 1 h. Dead cells were detected by adding 5 µl propidium iodide (PI; 3.2 mM in PBS) immediately prior to analysing the samples on the flow cytometer (FACScan; Becton Dickinson, USA) equipped with an argon laser exciting at 488 nm (Jacobs et al., 1983). Data was collected with logarithmic amplification and fluorescence intensity was displayed as arbitrary linear units. Fluorescence signals from 10,000 cells were acquired.

Staining of porcine embryonic VM tissue with anti-tyrosine hydroxylase, anti-monocyte/macrophage antibodies or the isolectin Bandereia Simplicifolia IB4. Whole heads from E26/E27 embryos were cut, immersion fixed in cold 0.1 M phosphate buffered 4% paraformaldehyde (pH 7.4) over night, followed by an incubation in a 20% sucrose-PBS solution for at least 24 h. The specimens were frozen, and four series of 40-µm-thick coronal sections were cut on a sliding microtome. Free-floating sections were stained.

For tyrosine hydroxylase and microglial-cell immunostaining, standard immunohistochemical methods were used (Duan et al., 1995). Endogenous peroxidase

activity was removed with a 3% H_2O_2/10% methanol solution, followed by a 1-h incubation in 5% swine serum. Sections were incubated over night in rabbit anti-TH (1:500; Pel-Freez, USA) or in mouse anti-human monocyte/macrophage (1:500; clone MAC387; IgG_1; Serotec, UK) mAbs diluted in PBS (pH=7.2-7.4) with 0.3% Triton X-100 (PBS-X) at room temperature. The latter antibody binds microglial cells and crossreacts with the porcine antigen (Serotec). The following day, sections were rinsed three times in PBS-X and incubated for 1 h with biotinylated swine anti-rabbit antibody (1:200; DAKO, Denmark) or with a polyclonal biotinylated horse anti-mouse IgG antibody (1:200; clone BA-2001; Vector Labs, USA), respectively. After rinsing in PBS-X, the Vectastain™ standard peroxidase ABC kit was used (Vector Labs). After 1 h, the sections were washed three times in PBS-X and visualised using a DAB (3.3′-diaminobenzidine) kit, (Vector Labs). Sections were mounted on gelatin slides, dehydrated using standard alcohol/xylene reagents, and cover-slipped with DePeX (BDH Labs, UK).

For staining with isolectin Bandereia Simplicifolia IB4 (BS-IB4), sections were treated with a 3% H_2O_2/10% methanol solution to block endogenous peroxide activity, followed by a 1 h incubation in 1% BSA blocking solution. Sections were incubated over night (4°C) in a 4 µg/ml solution of biotinylated BS-IB4 (L-2140; Sigma) diluted in PBS (pH=6.8) containing 0.2 mM $CaCl_2$ (PBS-Ca). On the following day, sections were washed in PBS-Ca three times and then incubated at room temperature with the AB solution contained in the Vectastain™ standard peroxidase ABC kit (Vector Labs). After rinsing in PBS-Ca, the sections were reacted with a DAB solution prepared using the Vector™ Dab kit (Vector Labs). Sections were mounted on gelatin slides, dehydrated using standard alcohol/xylene reagents, and cover-slipped with DePeX (BDH Labs).

Isolation of endothelial and VM cell membranes. Cells were washed once with PBS containing 1 mM PMSF and kept frozen at -70 °C until use. Following six cycles of freeze-thawing, cells were homogenised on ice in PBS/1 mM PMSF using a Dounce homogeniser (approximately ten strokes). Nuclei were pelleted by centrifugation for

15 minutes at 3,000 g, the supernatant was collected, and the pellet was resuspended in the same buffer and centrifuged again. This was repeated, and the pooled supernatants were ultracentrifuged at 100,000 g for 1 hour and 100 min in a Ti 75 rotor, the pellet resuspended in PBS/1 mM PMSF and overlayed on top of an equal volume of a 35 % (w/v) sucrose in PBS solution. Following centrifugation at 100,000 g for 1 hour without brake in a SW 40 rotor, the membranes were collected as a cloudy interphase, and again pelleted by centrifugation at 40,000 g for 1 h. VM and endothelial cell membrane proteins were solubilized in 10 mM Tris-HCl (pH=7.5) with 100 mM NaCl, 10 mM EDTA, and 1 % Igepal CA-630 (I-3021; Sigma) containing 1 mM PMSF (P-7626; Sigma), 1 μg/ml pepstatin A (P-4265; Sigma) and 1 μg/ml leupeptin (L-2023; Sigma). An equal volume of solubilized cell membrane proteins and 2 x reducing sample buffer were mixed and applied onto the gel.

SDS-PAGE and Western blotting. SDS-PAGE was run by the method of Laemmli (Laemmli et al., 1970) with 5 % stacking gels and 8 % resolving gels using a vertical Mini-PROTEAN II electrophoresis system (Bio-Rad, USA). Separated proteins were electrophoretically blotted onto Hybond™-C extra membranes (Amersham, UK) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). Protein gels were stained using a silver staining kit according to the instructions of the manufacturer (Bio-Rad). Following blocking for at least 2 h in 3 % BSA in PBS, the membranes were probed for 2 h at room temperature with peroxidase-conjugated *Bandereia Simplicifolia* isolectin B4 (L-5391, Sigma) diluted to 1 μg/ml in PBS (pH=6.8) containing 0.2 mM $CaCl_2$. The membranes were washed five times with PBS (pH=6.8), and bound isolectin was visualised by chemiluminescence using the ECL™ kit (Amersham). When membranes were probed with human serum, they were blocked over night with 3 % BSA in Tris-buffered saline with 0.05% Tween 20 (TTBS), incubated for 1 h at room temperature with non-diluted AB serum or anti-Gal antibody-depleted AB serum, washed five times with TTBS, and incubated for 1 h at room temperature with peroxidase-conjugated, $F(ab)'_2$ fragments of goat anti-human IgG or IgM antibodies (cat. nos. 109-036-098 and 109-036-129, respectively; Jackson ImmunoResearch

Labs.). Following washing in TTBS, antibody-binding components were detected using the ECL™ kit (Amersham).

For Example 4-7. Also for Example 1-3 unless specified otherwise.

Isolation and primary culture of porcine embryonic brain cells. Brains were dissected from 27-day-old pig embryos of the Pigham strain in Hank's balanced salt solution with 0.3 μM lazaroid U-83836E (Upjohn Inc., USA). Brain cells were isolated and grown in primary culture as described elsewhere (Brevig et al., 1997). Briefly, the meninges were removed, and the brains from each litter were pooled and cut into small pieces, which were transferred to brain-cell medium (Eagle's minimum essential medium with Earle's salts supplemented with heat-inactivated (56°C, 30 min) fetal calf serum (FCS), L-glutamine, amino acids, vitamins, penicillin, streptomycin, and sodium bicarbonate (all Life Technologies Inc., USA)). The tissue pieces were triturated with a flame-polished pipette, and the resulting cell suspension was filtered through an 80-μm-pore nylon mesh (Streno, Denmark) before diluting in brain-cell medium with 20% FCS (10 ml per brain). Aliquots of 5.0 ml cell suspension were added to poly-D-lysine (Sigma, USA) coated 50-ml culture flasks (Nunc, Denmark), and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was changed after 3 days, and again on day 6 and 9, now to brain-cell medium with 10% FCS. After 10-12 days, cultured brain cells were detached by gently sweeping across the bottoms of the culture flasks with a plastic pipette, transferred to 10-ml tubes (content of one flask to one tube), and centrifuged (100 g, 10 min). Supernatants were discarded, and the cells in the pellet were resuspended in 500 μl PBS with 2% FCS using a syringe with a needle of 0.5 mm internal diameter. The tubes were left for 15 min at room temperature to sediment cell clusters, and the supernatants with single cells in suspension were pooled and used for subsequent analyses.

Bandereia Simplicifolia isolectin staining of porcine embryonic brain cells. Suspended and adherent, cultured porcine embryonic brain cells were stained with *Bandereia Simplicifolia* isolectin B4 (BS-IB4) in order to determine the expression of the α-

galactosyl epitope by flow cytometry and light microscopy, respectively. Suspensions of 5.0×10^5 brain cells in 100 µl PBS were incubated with or without 3.0 µg FITC-conjugated BS-IB4 (Sigma) at 4°C for 30 min, washed twice in PBS (250 g, 5 min), and analysed by flow cytometry as described below. Brain cells adherent to a culture dish were fixed in 4% paraformaldehyde in PBS for 30 min, permeabilized by 1% Triton X-100 (Sigma) in Tris-buffered saline (TBS) for 15 min, and stained by incubation with biotinylated BS-IB4 (100 µg/ml TBS; Sigma) for 60 min, peroxidase-conjugated streptavidin (1:200 in TBS; DAKO, Denmark) for 60 min, and finally 0.05% 3,3'-diaminobenzidine (DAB; Sigma) in TBS for 15 min. Between the incubations, which were all at 20°C, the cells were thoroughly rinsed with TBS. After incubation with DAB, the cells were rinsed with TBS and distilled water, covered with Aquamount (BDH Laboratory Supplies, UK), and examined in an inverted light microscope.

Treatment of porcine embryonic brain cells with human serum and rabbit complement. Freshly isolated porcine embryonic brain cells were split into three aliquot suspensions (3.0×10^6 cells in 2.0 ml PBS), of which one received a treatment with human serum and rabbit complement: the cells were given 8.0 ml heat-inactivated human serum (pooled from blood type AB donors), incubated for 60 min at 4°C, washed in cold PBS (400 g, 5 min), resuspended in 5.0 ml Low-Tox-M rabbit complement (lyophilised content of one vial was reconstituted to 10 ml with PBS; Cedarlane Laboratories Ltd., Canada), incubated for 60 min at 37°C, and finally washed in PBS (400 g, 5 min). The three cell suspensions were cultured for 10 days in separate culture flasks as described above, and cells from one culture flask, not treated prior to culture, then received an identical treatment with human serum and rabbit complement. Cells from all three cultures were analysed by flow cytometry without prior staining, to determine the content of autofluorescent macrophages/microglial cells.

Isolation of human CD4 T lymphocytes. Mononuclear cells (lymphocytes and monocytes) from two healthy blood donors were isolated from buffy coats by flotation as earlier described (Brevig et al., 1997), cooled on ice for a few minutes, and mixed

with washed, paramagnetic Dynabeads M-450 CD4 (Dynal, Norway) to a bead-to-cell ratio of 0.6. The tubes were incubated head-over-tail at 4°C for 40 min and left in a magnetic particle concentrator (MPC; Dynal) for 3 min. The supernatants were discarded, and the positively isolated cells on the beads were washed five times in PBS with 2% FCS and resuspended in Detachabead CD4/CD8 reagent (1.0 µl per 2.8×10^5 beads; Dynal). After incubation head-over-tail at 20°C for 50 min, the tubes were given 5 ml PBS with 2% FCS and left for 3 min in the MPC. The positively isolated cells in the supernatants were transferred to new tubes and washed, in PBS with 2% FCS, once with the MPC and once by centrifugation (500 g, 10 min).

The purity of the isolated CD4 T lymphocytes was analysed by flow cytometry after incubating 5.0×105 cells in 100 µl PBS with 10 µl mouse anti-human CD3/4 FITC/RPE (Becton Dickinson, USA) at 4°C for 30 min and washing twice in PBS (250 g, 5 min). Isolated cells from individual A and B contained 99.1% and 99.7% double-positive cells, respectively.

Mixed lymphocyte-brain cell culture. A suspension of porcine embryonic brain cells, prepared as described above, were split into aliquots containing 3.0×10^6 cells in 190 µl PBS with 2% FCS, and each aliquot was given 1.0 ml of one of the following reagents: human IgG (10 mg/ml), human IgM (2.0 mg/ml), human anti-Gal (100 µg/ml), heat-inactivated human serum (pooled from blood type AB donors), or lymphocyte medium (90% RPMI 1640 with HEPES, 10% heat-inactivated FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2.0 mM L-glutamine; all Life Technologies). Human IgG, IgM, and anti-Gal were diluted in lymphocyte medium just prior to use. After 60 min at 4°C, the cells were washed in cold lymphocyte medium (400 g, 5 min) and resuspended in 1.0 ml Low-Tox-M rabbit complement (lyophilised content of one vial was reconstituted to 10 ml with lymphocyte medium). After 60 min at 37°C, the cells were washed (400 g, 5 min), resuspended in lymphocyte medium, and counted. About 15% of the brain cells were lost in the incubation and centrifugation steps. The cells were γ-irradiated (40 Gy), and 2.0×10^4 brain cells in 100 µl lymphocyte medium were

dispensed in wells of round-bottomed, 96-well plates (Nunc). Volumes of 100 µl lymphocyte medium containing 2.0×10^5 human CD4 T lymphocytes were added in triplicate to wells with brain cells. Wells receiving CD4 T cells and lymphocyte medium but not brain cells were used to determine the background proliferative activity of the CD4 T cells. Each well was given 1.0 µCi [^{3}H]thymidine (Amersham, UK) 24 h prior to cell harvest. After 3-7 days of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air, cells were harvested onto glass fibre filters and counted for 15 min in a 1450 MicroBeta Trilux β-counter (Wallac, Finland).

The brain-cell suspensions were analysed by flow cytometry without prior staining and after staining with propidium iodide (1.0 µg/ml PBS; Sigma) to determine the relative number of macrophages/microglial cells and viable cells, respectively. Samples given propidium iodide were analysed after 10 min in the dark at room temperature.

Flow cytometry. From each sample, 1.0×10^4 ungated events were acquired on a FACSort flow cytometer (Becton Dickinson). A forward-scatter threshold was set to exclude subcellular debris.

<u>For Example 8 (See also for Example 4-7 and Example 4 itself)</u>

Isolation and primary culture of porcine embryonic brain cells. Whole forebrains were dissected from 27-day-old pig embryos, dissociated, and grown in Eagle's minimum essential medium with Earle's salts supplemented with heat-inactivated (56°C, 30 min) fetal calf serum (FCS), L-glutamine, amino acids, vitamins, penicillin, streptomycin, and sodium bicarbonate (all from Life Technologies, USA) in poly-D-lysine (Sigma Chemical Co., USA)-coated culture flasks, as described in detail elsewhere (Brevig et al., 1999). After 10 or 12 days in culture (change of medium every third day), brain cells were detached and dissociated, washed (100 g, 10 min), and resuspended in phosphate-buffered saline (PBS, pH 7.4) with 2% FCS.

Purification of anti-Gal from human serum. As in Example 4 but only purification of human anti-Gal from serum pooled from more than 20 healthy, blood type AB donors using beaded agarose with porcine thyroglobulin (Sigma).

Isolation of human CD4 T lymphocytes. Human CD4 T lymphocytes were isolated from buffy coats of two healthy blood donors using Dynabeads M-450 CD4 (Dynal, Norway) and the corresponding Detachabead reagent (Dynal), as described (Brevig et al., 1997). The purity was analysed by flow cytometry after staining with mouse anti-human CD3/4 FITC/RPE (Becton Dickinson). Cells isolated from individual A and B contained 99.1% and 99.7% double-positive cells, respectively. Volumes of 100 µl medium containing 2.0×10^5 human CD4 T cells were added in triplicate to wells with brain cells or medium only. Each well was given 1.0 µCi [^{3}H]thymidine (Amersham, UK) 24 h prior to cell harvest. After 3–7 days of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air, cells were harvested onto glass fibre filters and counted for 15 min in a 1450 MicroBeta Trilux β-counter (Wallac, Finland).

Donor tissue isolation and pretreatment. Ventral mesencephalon (VM) was dissected from 27-day-old pig embryos of crown–rump length 19–21 mm. Tissue pieces from 16 embryos were pooled in 5.0 ml Dulbecco's modified Eagle medium (DMEM, cat. no. 41966-011; Life Technologies, Scotland) containing 0.1% porcine trypsin, type IX (cat. no. T-0134; Sigma) and 0.05% bovine deoxyribonuclease I, type IV (cat. no. D-5025; Sigma), incubated at 37°C for 20 min, washed five times in DMEM, and triturated in 1.0 ml DMEM containing 0.05% deoxyribonuclease using flame-polished glass pipettes of progressively smaller apertures. The resulting cell suspension was split into two aliquots, which were given 300 µg human anti-Gal in 600 µl DMEM (500 µg/ml) or 600 µl DMEM only (control). After 60 min at 4°C, the cell suspensions were washed in DMEM (200 g, 5 min), resuspended in 5.0 ml DMEM containing Low-Tox-M rabbit complement (lyophilized content of one vial was reconstituted to 10 ml, cat. no. CL3051; Cedarlane, Canada), and incubated for another 60 min at 37°C. The cells were then washed in DMEM (200 g, 5 min) and resuspended in

DMEM to a concentration of 1.4×10^5 cells/μl. Anti-Gal-pretreated and control-pretreated cell suspensions contained 90% and 92% viable cells (trypan-blue negative), respectively.

In context herewith, it should be noted that the preceding paragraph is a preferred embodiment of the kit according to the invention.

Transplant recipients and induction of hemiparkinsonism. Twenty-two male Lewis rats weighing 230–340 g were anesthetized with Hypnorm (fentanyl citrate 0.20 mg/kg and fluanisone 6.3 mg/kg, i.p.; Janssen, Belgium) and Dormicum (midazolam 4.2 mg/kg, i.p.; Hoffmann–La Roche, Switzerland), and placed in a Kopf stereotaxic frame with the toothbar set at 3.9 mm below the interaural line. Unilateral lesions of the dopaminergic nigrostriatal pathways were produced by injecting 4.0 μl sterile saline containing 32 mM 6-hydroxydopamine HCl (Sigma) and 1.0% ascorbate at 1.0 μl/min at the following coordinates relative to *bregma* and *dura mater*: posterior 2.8 mm, right 2.0 mm, and ventral 8.4 mm. To relieve postoperative pain, Temgesic (buprenorphinum 0.10 mg/kg, i.m.; Reckitt & Colman, UK) was given upon completion of surgery. After 6 weeks, circling behavior of the animals in response to D-amphetamine sulphate (2.5 mg/kg, i.p.; Sigma) was tested for 90 min in automated rotometer cylinders, as described (Ungerstedt et al., 1970), and 16 rats, all with more than 8 net turns per min ipsilateral to the lesion, were assigned to two groups (n = 8) of equal rotational rates.

Intrastriatal transplantation. Seven weeks after injection of 6-hydroxydopamine, rats received 3.0 μl DMEM with anti-Gal-pretreated or control-pretreated VM cells at 1.0 μl/min at the following coordinates relative to *bregma* and *dura mater*: anterior 0.7 mm, right 2.6 mm, and ventral 4.7 mm (toothbar set at 2.5 mm below the interaural line). Animals were anesthetized and pain relieved as described above. Rotational response to D-amphetamine sulphate was assessed 3, 7, and 10 weeks posttransplantation, as described above, and Mann–Whitney statistical tests ($p = 0.03$)

were performed for each time point (two-tailed p values were estimated using the z statistic).

Tissue processing and immunohistochemistry. All rats were sacrificed 10 weeks after transplantation by an overdose of pentobarbital and perfused transcardially with 4% paraformaldehyde in 0.15 M phosphate buffer (pH 7.4). After perfusion, brains were immersed in the same fixative for 60 min, cryoprotected overnight in 20% sucrose in 0.15 M phosphate buffer (pH 7.4), and frozen in gaseous CO_2. Four parallel series of 30-µm-thick cryostat sections were cut and mounted on gelatine-coated glass slides. Whole series of sections were incubated for 30 min with 10% FCS in Tris-buffered saline (TBS, pH 7.4), and then incubated overnight at 4°C with one of the following primary antibodies in TBS with 1% Triton X-100 (Sigma): mouse anti-cow neurofilament-70 IgG_1 (1:1000; clone DP.5.112; purchased from Dr. L. Soriano, France (soriag@aol.com)), polyclonal rabbit anti-rat tyrosine hydroxylase (1:250; Pel-Freez Biologicals, USA), or mouse anti-rat CD45 IgG_1 (1:100; clone MRC OX-1; Serotec, UK). The neurofilament and tyrosine-hydroxylase antibodies are known to crossreact with their respective porcine antigens, but the neurofilament antibody does not react with rodent neurofilament. Following rinsing for 3 × 15 min in TBS with 1% Triton X-100, the sections were incubated with biotinylated sheep anti-mouse Ig (1:200 in TBS with 1% Triton X-100; Amersham) or biotinylated donkey anti-rabbit Ig (1:200 in TBS with 1% Triton X-100; Amersham) for 60 min at 20°C, rinsed 3 × 15 min in TBS with 1% Triton X-100, treated for 30 min with 0.06% H_2O_2 in methanol to block endogenous peroxidase, rinsed 3 × 15 min in TBS with 1% Triton X-100, and incubated for 60 min with peroxidase-conjugated streptavidin (1:200 in TBS with 10% FCS; DAKO). After a repeated rinse, 0.05% 3,3'-diaminobenzidine (Sigma) in TBS with 0.009% H_2O_2 was applied to the sections until a brown precipitate developed. The sections were finally rinsed in distilled water, dehydrated in alcohol, cleared in xylene, and coverslipped in Depex mounting medium (BDH Laboratory, UK).

Example 1 – Macrophages/microglial cells become autofluorescent in culture, astrocytes do not.

Unstained brain cells from 28-day-old pig fetuses were analysed with flow cytometry directly after isolation (Fresh) or after 14 days in primary culture (Cultured) (Fig. 1). Plots A and B show the green and orange fluorescence of the cells. Histograms C and D show the frequency of cells as a function of their green fluorescence, and include identical markers (M1) and the percentage of the total cell number present within the markers. Plot B shows regions set to define autofluorescent (R1) and non-autofluorescent (R2) cell populations, and plots E and F show the size (forward scatter) and granularity (side scatter) of the autofluorescent (R1) and non-autofluorescent (R2) cells.

In flow cytometric analyses of unstained cells, freshly isolated porcine fetal brain cells came out as a single population of cells without autofluorescence, while primary cultures of these cells, prepared to favour growth of astrocytes and macrophages/ microglial cells, consisted of two cell populations: a larger population of non-autofluorescent cells and a smaller population of autofluorescent cells. The autofluorescent cells were more granular than the non-autofluorescent cells (Fig. 1).

Brain cells isolated from 28-day-old fetal pig brains were grown for 14 days in primary culture. Cells were immunostained using FITC-conjugated (green fluorescence) or RPE-conjugated (orange fluorescence) antibodies (Fig. 2). The isotype control (IC) IgG_1 is the negative control for the CD18 and CD56 stainings, and the isotype control IgG_{2a} is the negative control for the CD44 and CD4 stainings. Cells were fixed and permeabilized prior to staining for GFAP. The GFAP control is a sample that received secondary antibody only. Regions were set in plots G and H to compare the GFAP staining of autofluorescent and non-autofluorescent cells. Mean green fluorescence intensity in arbitrary units: $G_{R1} = 151$, $G_{R2} = 199$, $H_{R1} = 24$, and $H_{R2} = 131$. Similar results were obtained with cultured brain cells from 35-day-old fetal pigs.

Only the autofluorescent cells were CD18 positive, and only the non-autofluorescent cells were CD56 positive, while both populations were CD44 positive. Antibodies against CD4 only stained some of the autofluorescent cells, and only very faintly. The non-autofluorescent cells were stained more intensively by antibodies against the astrocyte marker glial fibrillary acidic protein (GFAP) than the autofluorescent cells (Fig. 2).

Cultured brain cells isolated from 28-day-old pig fetuses were incubated with fluorescein-labelled *E. coli* (Fig. 3). The figure shows phagocytosis of *E. coli*. Plot A shows cells from a culture that received fluorescein-labelled *E. coli* (green fluorescence) 30 min prior to flow cytometry, while plot B shows cells from an untreated culture. The confocal laser microscopy image shows a cross section of a cell stained with the lipophilic and fluorescent DiI prior to incubation with fluorescein-labelled *E. coli* and dual-channel scanning. The red pseudocolour represents membranes and other lipids stained with DiI (rhodamine optical filter sets) and the yellow pseudocolour represents fluorescein-labelled *E. coli* (fluorescein optical filter sets). Scale bar: 10 μm. Flow cytometric analysis of brain-cell cultures incubated with fluorescein-labelled *E. coli* showed that the autofluorescent cell population split into two along the green-fluorescence axis of the dot plot, while the non-autofluorescent cell population remained as one (Fig. 3). When examined in a fluorescence microscope, the green fluorescence of the bacteria was not associated with the plasma membrane (not shown). In cultures stained with the lipophilic and fluorescent DiI and subsequently incubated with fluorescein-labelled *E. coli*, confocal laser microscopy showed that the majority of the cells only were stained by DiI. Some cells had, however, fluorescent *E. coli* within cell borders (Fig. 3).

Porcine fetal brain cells, cultured under conditions that favour growth of astrocytes and macrophages/microglial cells (Castellano et al., 1991), consisted of both autofluorescent cells and non-autofluorescent cells, when analysed by flow cytometry. Autofluorescence is a known property of macrophages (Havenith et al., 1993; Nicod et

al., 1989), and might be due to endogenous flavoproteins (Aubin et al., 1979; Benson et al., 1979), which are excitable by the 488-nm lasers used in this study, and can emit light in the green and orange ranges of the spectrum. The autofluorescent cells had high granularity, again typical of macrophages. To determine whether the autofluorescent cells were macrophages, we stained the cultured cells with antibodies known to react with astrocytes or macrophages, and tested the cells for phagocytic activity. The autofluorescent cells were $CD18^{+}CD44^{+}CD56^{-}GFAP^{-}$, strongly suggesting that these cells indeed were macrophages or microglial cells. Since no marker was available to distinguish between macrophages and microglial cells, we refer to these cells as macrophages, as this is commonly done at this early stage of brain development (Perry et al., 1988). The non-autofluorescent cells were $CD18^{-}$ $CD44^{+}CD56^{+}GFAP^{+}$, suggesting that these cells were astrocytes. After incubation of cultured brain cells with fluorescein-labelled *E. coli*, the autofluorescent cell population split into two along the green-fluorescence axis of the dot plot, and fluorescence microscopy and confocal laser microscopy revealed that the fluorescent bacteria were located within the cells, as strong evidence of phagocytic activity by the autofluorescent cells. Based on their phenotypic characteristics and phagocytic activity, we conclude that the autofluorescent cells were macrophages/microglial cells, while the non-autofluorescent cells were astrocytes.

Example 2 – Macrophages/microglial cells induce proliferation of human T lymphocytes, astrocytes do not

Brain cells isolated from 28-day-old fetal pig brains were grown for 14 days in primary culture and flow sorted after staining with RPE-conjugated anti-CD56 (orange fluorescence) (Fig. 4). **A.** *Purity and viability.* Cells were analysed by flow cytometry without further staining to determine the purity (region R1 was set to include the CD56-negative macrophages), and after staining with propidium iodide (orange fluorescence) to determine the viability. The numbers given are the percentages of cells within region R1. The viability of the astrocyte population was 91% (determined by trypan-blue exclusion). **B.** *Ability to induce human T-cell proliferation.* Allogeneic

mononuclear blood cells (MBC; H-8053), sorted astrocytes, and sorted macrophages were γ-irradiated (50 Gy) to halt their proliferation and mixed with lymphocytes from either of two human individuals (H-3970 and H-3977) (3 wells per combination). As a negative control, the lymphocytes were incubated without stimulators (12 wells per combination). The proliferative activity was determined by [^{3}H]thymidine incorporation after 4 days of co-culture. Bars represent means and standard errors of means.

The proliferative activity of the lymphocytes was higher when co-cultured with sorted macrophages than when co-cultured with allogeneic mononuclear blood cells. In co-cultures of sorted astrocytes and lymphocytes from either of the two human individuals, the proliferative activity was slightly higher in wells with lymphocytes and astrocytes than in wells with lymphocytes only, but the difference was only statistically significant for one of the individuals. An ANOVA was done for each individual: $F_{H\text{-}3970}$ (df 3,17) = 139, $p = 3.8 \times 10^{-12}$ and $F_{H\text{-}3977}$ (df 3,17) = 216, $p = 1.0 \times 10^{-13}$. Stimulator cells were compared with medium in one-sided, unpaired *t* tests and all *p* values were less than 0.001, except when comparing the stimulatory ability of astrocytes and medium on lymphocytes from individual H-3977 ($p = 0.08$).

By flow sorting, a highly enriched macrophage population (increase in macrophages from 7.6% to 91%) and a highly purified astrocyte population (more than 99% astrocytes) was obtained, and only a few percent of the sorted cells were dead. The sorted macrophages were strong inducers of human T-cell proliferation, even stronger than allogeneic mononuclear blood cells. The sorted astrocytes were only weak stimulators, and with one of the two human individuals, the T-cell proliferation was not significantly higher than the background proliferative activity.

Example 3 - The α-galactosyl epitope is expressed on macrophages/microglial cells and not on neurons.

Pig embryonic VM cells, including neurons, glia, and endothelial cells were analysed by flow cytometry for expression of α-galactosyl epitopes using biotinylated *Bandereia Simplicifolia* isolectin B4 (BS-IB4) (Fig. 5A). A slight shift in fluorescence of less than one log was noted for the bulk population of brain cells when compared to the control. (FITC-conjugated avidin alone). This should be compared to BS-IB4 staining of PLMECs where a shift to the right on the logarithmic scale of at least two ten log steps was seen (Fig. 5B).

In order to verify these flow cytometry data, Western blot analysis was done on BS-IB4 reactive, solubilized glycoproteins isolated from cell membranes of VM, PLMEC and ECV cells following separation on 8% SDS-PAGE, in order to detect possible proteins carrying α-galactosyl epitopes. PLMECs were shown to express high levels of α-galactosyl epitopes (Fig. 6); epitopes that were carried on several different protein backbones as indicated by staining of components with molecular weights ranging from high (> 220 kDa) to low (≤ 30 kDa) mass. Detection was accomplished by chemiluminescence and the two blots represent 5 and 10 second-exposures, respectively. Upon longer exposure times (> 10 seconds), we saw weak staining in the VM lane of a component of approximately 150 kDa size. Under these conditions, a component of similar size and staining intensity was seen among the ECV membrane proteins (Fig. 6).

The distribution of α-galactosyl epitopes on individual VM cell populations was examined using BS-IB4 immunohistochemistry. Fig. 7 shows a saggital section of the VM of a 27 day-old pig embryo stained immunohistochemically for tyrosine hydroxylase, which is the rate-limiting enzyme for dopamine production in dopaminergic neurons. Cell bodies and some axonal fibres are seen (Fig. 7A). Low power magnification of a coronal section of a 27 day-old pig embryo stained immunohistochemically using peroxidase-conjugated BS-IB4 isolectin (Fig. 7B).

There is intense staining of embryonic brain microvascular endothelium (Fig. 7B), and a somewhat less intense staining of microglial cells/tissue resident macrophages as seen at higher magnification (Fig. 7C). No prominent staining was found on neurons or glial cells. Staining of cells similar in morphology to the BS-IB4 stained cells in panel C, was seen with a mouse mAb specific for monocytes/tissue macrophages, which also binds porcine microglial cells (Fig. 7D).

It is conceivable that the staining of VM membrane proteins, which was of approximately the same intensity as the staining of ECV proteins, may be due to recognition of determinants related to α-Gal. However, isolectin-histochemistry indicated that the majority of the α-Gal expression is restricted to endothelial cells and microglial cells/macrophages, and not to neurons and astrocytes, in fetal porcine brain tissue (Fig. 7). BS-IB4 reactivity in the adult mouse brain has also been shown to be confined to capillaries (Peters et al., 1979). Therefore, a more-likely explanation for the low-level expression in the Western blot experiments, is that the α-Gal expressing cells constitute only a small fraction of the total number of cells in the VM.

Example 4 - Purification of antibodies from human serum

Human IgG, IgM, and anti-Gal were purified from serum pooled from more than 20 healthy, blood type AB donors using beaded agarose with goat anti-human IgG (Fc specific; Sigma, USA), goat anti-human IgM (μ-chain specific; Sigma), or porcine thyroglobulin (source of α-galactosyl epitope; Sigma), respectively. Five ml of slurry (2.5 ml of packed beads) were poured into a column of 10 mm diameter and washed with PBS. Heat-inactivated human serum (40 ml) was applied at 0.5 ml/min using a peristaltic pump, the agarose was washed with several column volumes of PBS, and bound protein was eluted with 0.1 M glycine/HCl (pH=2.5) at 1.0 ml/min. One-millilitre fractions were collected in tubes containing 0.1 ml neutralising buffer (1.5 M Tris/HCl, pH=8.8). The absorption at 280 nm was read spectrophotometrically, and contents of tubes containing protein were pooled, dialysed against 1% PBS, lyophilised, and resuspended in distilled water (1/100 of eluted volume). The protein

concentration was determined using a BCA Protein Assay Reagent Kit (Pierce, USA) according to the manufacturer's instructions.

The purity of the IgG and IgM preparations was analysed by SDS-PAGE: 4.0 μg of each sample was boiled in SDS reducing or non-reducing buffer for 5 min and loaded onto ready-made, 4-15% continuous gels (Bio-Rad, USA). After electrophoresis, the gels were stained using a silver-staining kit (Bio-Rad) according to the manufacturer's instructions. No IgM bands appeared in the IgG lanes, but weak IgG bands were present in the IgM lanes.

The α-galactosyl-epitope reactivity of the antibodies purified from human serum using porcine thyroglobulin was assessed by incubating 5.0×10^5 Raji cells (human Burkitt lymphoma cell line) or Raji cells transfected and selected to stably express the porcine α1,3-galactosyl transferase gene (Raji-GT cells) in 100 μl PBS with or without 10 μg of the purified antibodies. After 60 min at 4°C, the cells were washed once in PBS (250 g, 5 min), incubated with rabbit anti-human IgG $F(ab')_2$ FITC (1:10; DAKO, Denmark) or rabbit anti-human IgM $F(ab')_2$ FITC (1:10; DAKO) at 4°C for 60 min, washed twice in PBS (250 g, 5 min), and analysed by flow cytometry. The expression of the α-galactosyl epitope on Raji-GT cells was verified by staining Raji-GT cells and Raji cells with 3.0 μg FITC-conjugated BS-IB4. Transfection and selection of the Raji-GT cell line has been described by Kumagai-Braesch et al. (1998).

Example 5 – Macrophages/microglial cells cultured from embryonic pig brain tissue express the α-galactosyl epitope

Porcine embryonic brain cells were grown in primary culture with a high serum content in the medium, conditions which produce autofluorescent macrophages/ microglial cells and non-autofluorescent astrocytes, as shown in Example 1.

Cultured cells were analysed unstained and after staining with FITC-conjugated *Bandereia Simplicifolia* isolectin B4 (BS-IB4 FITC) by flow cytometry. The dotplots show the intensity of green (FL1) and orange (FL2) fluorescence (see Fig. 8), and contain a line that separates autofluorescent macrophages/microglial cells (m) from non-autofluorescent astrocytes (a). Staining resulted in an increase in green-fluorescence geometric means of 1167 units for macrophages/microglial cells and 16 units for astrocytes. Thus, macrophages/microglial cells express the α-galactosyl epitope, but it is not clear whether the weak staining of astrocytes reflects low α-galactosyl-epitope expression or unspecific binding of BS-IB4 FITC. Note the logarithmic scales on the axes. The photomicrograph (300×) shows adherent, cultured brain cells which were fixed, permeabilized, and stained with biotinylated BS-IB4 and peroxidase-conjugated streptavidine. The BS-IB4-positive cells are rounded with the DAB staining product concentrated on the cell membrane.

As shown by McKenzie et al., 1995, the α-galactosyl epitope expression may change when porcine tissue is cultured, but macrophages/microglial cells from embryonic pig brain maintained their α-galactosyl epitope expression in culture (Fig. 8).

Example 6 - Human serum and rabbit complement remove macrophages/microglial cells from cultured and freshly isolated porcine embryonic brain cells

Dissociated brain tissue was split into aliquots, of which one was treated with human serum and rabbit complement prior to culture for 10 days, and another received an identical treatment after culture. These cells and untreated, cultured cells were analysed by flow cytometry without prior staining to compare the content of macrophages/microglial cells. In Fig. 9, the histograms show the cell frequency as a function of green-fluorescence intensity (FL1), and contain identical markers (M1) to define the autofluorescent macrophages/microglial cells and the percentage of the cells that are within the markers. Both when applied to freshly isolated cells and to cells grown in primary culture, treatment with human serum and rabbit complement reduced the relative number of macrophages/microglial cells.

Example 7 - Treatment of porcine brain cells with anti-Gal and complement reduces the content of macrophages/microglial cells and their ability to induce human CD4 T-lymphocyte proliferation

Raji cells (Raji) and Raji cells transfected to express porcine α1,3-galactosyl-transferase (and thus the α-galactosyl epitope; Raji-GT) were incubated with the antibodies against the α-galactosyl epitope (purified from normal serum as described in Example 4; in Fig. 10 referred to as aGal) or no antibodies and stained with either FITC-conjugated anti-human IgG (aIgG) or FITC-conjugated anti-human IgM (aIgM). The expression of the α-galactosyl epitope on Raji-GT cells was verified by staining these and Raji cells with FITC-conjugated BS-IB4. The purified antibodies contained both IgG and IgM reactive with Raji-GT cells, but not with Raji cells. Unspecific staining of Raji-GT cells by aIgG and aIgM was minimal, and the purified antibodies thus contained both IgG and IgM reactive with the α-galactosyl epitope (see Fig. 10).

Porcine embryonic brain cells were grown in primary culture for 12 days, dissociated, and incubated with human IgG (circle in Fig. 11), human IgM (square in Fig. 11), human anti-Gal (triangle in Fig. 11), human serum (diamond in Fig. 11), or medium (filled circle in Fig. 11). Human IgG, IgM and anti-Gal were prepared as described in Example 4. After 60 min at 4°C, the cells were washed, resuspended in medium with rabbit complement, incubated at 37°C for 60 min, washed again, γ-irradiated to halt their proliferation, and mixed with highly purified human peripheral blood CD4 T cells. CD4 T cells were also grown in absence of brain cells to determine their background proliferative activity (cross in Fig. 11). Proliferation was determined as incorporation of tritiated thymidine, and means of triplicate cultures are given in counts per minute (cpm) in Fig. 11. Bars represent one SEM. IgG and IgM reactive with the α-galactosyl epitope were present in the anti-Gal preparation (Fig. 10). The effect of the different brain-cell pretreatments on the content of macrophages/microglial cells and cell viability is given in Table 1 below.

Table 1. Content of macrophages/microglial cells and cell viability of pretreated porcine embryonic brain cells for co-culture with human CD4 T lymphocytes		
Pretreatment	Macrophages/microglial cells (%)	Viable cells (%)
Human IgG	1.4	83.2
Human IgM	1.1	87.5
Human anti-Gal	0.8	87.1
Human serum	1.1	89.6
Medium	3.4	87.5

Cultured porcine embryonic brain cells treated with human IgG, human IgM, human anti-Gal, or human serum and rabbit complement did not induce proliferation of human CD4 T cells, while brain cells given a control treatment with medium and rabbit complement induced a significant proliferative response in human CD4 T cells (Fig. 11).

Even though human IgG, human IgM, human anti-Gal, and human serum were not equally effective at removing macrophages/microglial cells (Table 1), the proliferative response of the CD4 T cells to the remaining brain cells was not higher than background activity (Fig. 11). This suggests the presence of a threshold, below which the macrophages/microglial cells are too few to elicit a T-cell response in vitro, and possibly in vivo.

An in-vitro model was used to study the human CD4 T-cell response to porcine embryonic brain cells (Fig. 12). The brain cells were grown in primary culture prior to co-culture with human CD4 T cells, because only cultured cells, and not freshly isolated cells, will induce proliferation of human T cells (Brevig et al., 1997). Culturing under the conditions described herein, like intracerebral transplantation (Mason et al., 1986; Duan et al., 1995), upregulates the expression of MHC antigens (Brevig et al., 1999).

Fig. 12 shows a summary of the effect of anti-Gal and complement (C) on dissociated porcine brain tissue in the clinical situation and in the in-vitro model used by the inventors. Dissociated brain tissue from pig embryos contains neurons (N), astrocytes (A), macrophages/microglial cells (M), and endothelial cells (E). The latter two cell types express the α-galactosyl epitope (α-Gal), and their removal prior to transplantation will greatly reduce the potential of the donor tissue to upregulate MHC antigens, particularly class II, once implanted. Primary culture in medium with a high serum content favours growth of astrocytes and macrophages, and upregulates the expression of MHC antigens. Dissociated porcine brain tissue cultured under these conditions can thus be used to study the human T-cell response to porcine neural xenografts in vitro.

The approach of the inventors, which is shown in Figure 12, was (i) to target the α-galactosyl epitope, because it is expressed on macrophages/microglial cells, but not on neurons, in fresh porcine brain tissue (Sumitran et al., 1999), and (ii) to use antibody- and complement-mediated lysis, since even macrophages/microglial cells within small cell clusters can be removed by this mechanism.

Pretreatment of dissociated porcine brain tissue with anti-Gal and complement probably also removes endothelial cells, because the α-galactosyl epitope is abundantly expressed on endothelial cells in embryonic pig brain (Sumitran et al., 1999), and porcine aortic endothelial cells are sensitive to lysis by anti-Gal and complement (Vaughan et al., 1994). In culture, porcine aortic endothelial cells constitutively express class I, but not class II, MHC antigens, and induce proliferation of human CD8, but not CD4, T cells (Murray et al., 1994). After culture with human tumour necrosis factor-α, which may be present in neural grafts due to surgically induced inflammation, porcine aortic endothelial cells also express MHC class II antigens and induce proliferation of human CD4 T cells (Batten et al., 1996). Removal of endothelial cells may thus be beneficial for the survival of neural xenografts, but it is not known whether donor-derived endothelial cells are necessary for xenograft neovascularization in humans. However, in an allogeneic rat model, whole-suspension

grafts and grafts depleted of microglial cells and endothelial cells developed identically in terms of neovascularization and colonisation by host microglial cells (Pennell and Streit, 1997).

Example 8 - Effects of the pretreatment on survival and function of porcine neural xenografts in rats

Immunocompetent rats with 6-hydroxydopamine-induced hemiparkinsonism received dissociated VM tissue pretreated with anti-Gal and complement or medium and complement (control), and the functional effects of the grafts were followed over time until sacrifice of the animals at 10 weeks posttransplantation (Fig. 13). In 5 of 8 rats receiving VM cells pretreated with anti-Gal and complement, a reduction of amphetamine-induced circling behavior by more than 50% occurred during the 10-week follow-up. *The lesion in this recipient progressed beyond the time of transplantation. †Large graft with several clusters of dopaminergic neurons (photomicrographs shown in Fig. 14). ‡Small graft with few dopaminergic neurons. None of the 8 rats receiving control-pretreated VM cells displayed this degree of improvement. Histological analysis revealed a small graft, densely infiltrated by macrophages and containing only a few dopaminergic neurons, in one animal in the control group (not shown), and a large graft containing several clusters of dopaminergic neurons in one animal receiving VM cells pretreated with anti-Gal and complement (Fig. 14). The rest of the animals had completely rejected their grafts. (A) Shows an even distribution of porcine neurofilament in the graft. (B) Section stained for tyrosine hydroxylase, a marker for dopaminergic neurons, with stained cell bodies clustered and located at the graft–host interface, which is common for intrastriatal grafts of dissociated VM tissue. (C) Dopaminergic cell bodies at high magnification (arrows). (D) Shows infiltration by host leukocytes (staining for CD45) around a vessel at the graft–host interface (arrow). Gr, graft. Str, host striatum. Scale bars: 250 μm (A and B) and 25 μm (C and D).

Previous studies disagree as to whether non-pretreated porcine embryonic VM grafts induce behavioral effects in hemiparkinsonian, immunocompetent rats (Barker et al. 2000; Larsson et al., 2000; Galpern et al., 1996), emphasizing the importance of using identical aliquots of donor material for testing donor-tissue pretreatments. Functional benefit from the grafts pretreated with anti-Gal and complement was observed 7 weeks posttransplantation, which is consistent with other studies (Barker et al., 2000; Larsson et al., 2000; Galpern et al., 1996; Huffaker et al., 1989), in which functional benefit from non-pretreated porcine embryonic VM grafts occurred after 7–9 weeks in immunosuppressed rats.

Two studies have previously attempted to reduce the immunogenicity of brain tissue. Bartlett et al. upregulated MHC antigens on murine embryonic neural cells by culture with interferon γ, and showed that depletion of MHC class-I positive cells by flow sorting improves graft survival in brains of allogeneic mice (Bartlett et al., 1990). As a pretreatment of dissociated porcine embryonic brain tissue, Pakzaban et al. masked MHC class I antigens by divalent antigen-binding fragments of antibodies, and found improved graft survival in rat brain (Pakzaban et al., 1995). Masking MHC class I antigens on porcine embryonic brain cells reduces the proliferative response of human CD8 T cells by about 50% *in vitro* (DerSimonian et al., 1999). MHC antigens are, however, not optimal targets for donor tissue pretreatments, because these molecules are present at very low levels in fresh brain tissue (Lampson, 1995) and upregulated after implantation (Widner et al., 1988; Duan et al., 1995; Mason et al., 1986) (Fig. 12).

We conclude that pretreatment with anti-Gal and complement reduces the immunogenicity of porcine neural tissue. In order to estimate what can be expected from this pretreatment in the clinical situation, it will be necessary to transplant pretreated porcine neural tissue into the brains of Old-World monkeys, because these primates can be expected to have cellular and humoral immune responses comparable with those in humans, particularly the contribution of direct and indirect T-cell responses to the rejection of porcine grafts (Brevig et al., 2000). The pretreatment with

anti-Gal and complement might be a valuable alternative or supplement to immunosuppression, and it can, unlike current immunosuppressive drugs, be expected to have no side effects. Moreover, like immunosuppression, it can be combined with other strategies to reduce cellular immune rejection, for example, MHC class-I masking (Pakzaban et al., 1995) and Sertoli-cell co-grafting (Sanberg et al., 1996), and with strategies to reduce the complement response (Cozzi et al., 1995).

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